THE SIGNIFICANT RÔLE OF FRUCTOSE-1,6-DIPHOSPHATE IN THE REGULATORY KINETICS OF PHOSPHOFRUCTOKINASE

Kevin HOOD* and Michael R. HOLLAWAY
Department of Biochemistry, University College London. Gower Street, London WC1E 6BT, England

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1. Introduction

It is generally accepted that phosphofructokinase (EC 2.7.1.11, here represented as PFK) plays a key role in the regulation of glycolytic flux (for a review see ref. [1]). Therefore a number of different ligands that influence PFK activity, including the inhibitors ATP and citrate, and the activators AMP, F6P and FDP, represent candidates for the modulation in vivo of the rate of glycolysis (see ref. [2]).

The present study is concerned mainly with the mechanism for FDP 'activation' of PFK. We follow the time-course of the reaction by a method avoiding the use of coupling enzymes and with enzyme concentrations approaching that in vivo. The results indicate a crucial role for FDP in activating PFK under conditions simulating those in vivo, either by increasing the rate of hysteretic transitions, or by inducing a conformational change in a given enzyme molecule to give an increased catalytic activity at the other, non FDP-liganded sites.

2. Materials and methods

Rabbit skeletal muscle PFK, with a specific activity of 180–200 μmol/min/mg in the assay described by Dyson and Noltman [3], was prepared by a modification of the method of Kemp and Forest [4].

ATP, F6P and FDP were the best grades available from Boehringer (London) Corp., London W.5. Buffer components and other chemicals were AnalaR reagent grade.

Time-courses of the enzymic reaction were followed spectrophotometrically at 400 nm, at 25°C and pH 7.1–7.2 by using an indicator-coupled assay with *p*-nitrophenol as the chromophoric proton acceptor (cf. the assay of Hofer [5]). The assay mixture contained: 15 mM triethanolamine HCl, 67 mM KCl, 1 or 10 mM $MgCl_2$ as indicated and 0.08 mM *p*-nitrophenol, adjusted to pH 7.1–7.2, together with the indicated concentrations of substrates and effectors. The buffering capacity of this mixture was sufficient to limit the pH change during the reaction to less than 0.1 units, so the absorbance changes could be considered to be directly proportional to the extent of proton release.

An aliquot of enzyme (0.7–1.0 mg/ml in a 10 mM P_i buffer, pH 7.4, containing 4 mM dithiothreitol and 0.5 mM EDTA at 25°C, was added to the assay mixture containing all components except F6P. The mixture was then incubated for 4 min at 25°C before initiation of the reaction with F6P (the shapes of the time-courses were independent of preincubation time after a 2-min period). Changing the order of addition of reaction components profoundly affected the observed time course: for example initiation of the reaction with enzyme did not give rise to the lag periods given by the F6P initiated reactions.

p-Nitrophenol did not interfere with the reaction since (a) incorporation of different concentrations of *p*-nitrophenol into the assay did not change the observed time-course (after making the appropriate amplitude correction) and (b) identical time-courses were obtained using a pH-stat assay and the spectrophotometric assay at the same pH values.

*Present address: National Institute for Medical Research, Mill Hill, London NW7, England.

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With enzyme concentrations less than 10 μg/ml the absorbance changes were followed by using a Perkin–Elmer 124 UV-visible spectrophotometer but with higher enzyme concentrations a Durrum-Gibson stopped-flow spectrophotometer was employed. In the latter case the procedure was essentially the same as described above except that the triethanolamine concentration was 5 mM.

3. Results

3.1. *Lags in time-courses of the PFK-catalysed reaction at low and high enzyme concentrations*

Figure 1 shows a typical time-course for the PFK-catalysed reaction at pH 7.14 in which the enzyme was preincubated in the assay cuvette with MgATP and citrate and the reaction initiated by addition of F6P. Following a lag-period of several minutes the rate of the reaction rapidly approached a maximum (designated v_a in fig.1) in a strongly autocatalytic process. Qualitatively similar results have been reported by Colombo et al. [6] although they employed an assay procedure involving coupling through the aldolase reaction (see the Discussion section). The maximum value of the rate was the same as that in a corresponding assay initiated by addition of enzyme, in which case no lag periods were observed on the time-scale of these experiments. In the F6P-initiated assays the lag period increased, and the v_a value decreased with increasing concentrations of ATP or citrate.

It was important to determine whether the striking

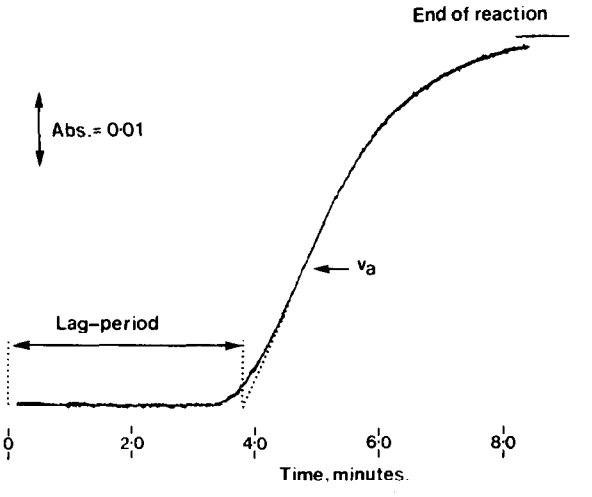

Fig.1. A time-course of the reaction catalysed by PFK in the presence of citrate. Enzyme (7.7 μg/ml) was incubated in a solution containing 1.5 mM ATP, 1.0 mM sodium citrate, 10 mM $MgCl_2$, 67 mM KCl and 0.08 mM *p*-nitrophenol, pH 7.14, and the reaction initiated by addition of F6P to a final concentration of 0.4 mM. Other conditions are as described in the text. The vertical scale corresponds to a decrease in absorbance at 400 nm. The empirical procedure for measuring the length of the lag-period is shown and the v_a value corresponds to the maximum slope of the curve.

hysteretic behaviour would also be observed at enzyme concentrations approaching that in rabbit skeletal muscle. From the photographs of time-courses recorded using the stopped flow apparatus (fig.2) it can be seen that the hysteretic response persisted at enzyme concentrations up to 0.31 mg/ml, although

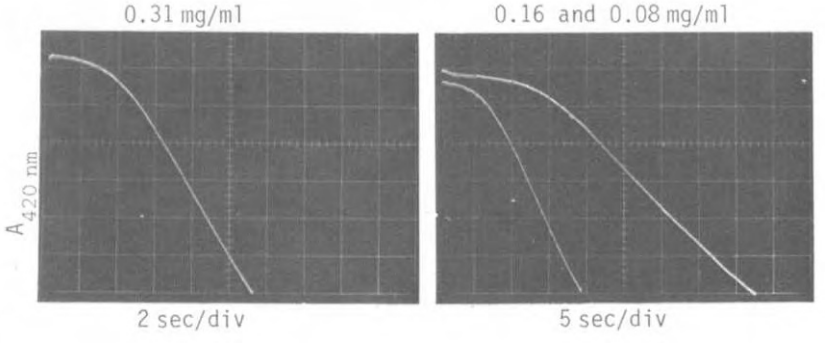

Fig.2. Stopped-flow time courses of the PFK-catalysed reaction at physiological enzyme concentrations. In each reaction, enzyme was in one syringe with ATP (3 mM) and citrate (2 mM). The F6P solution (2 mM) was in the other syringe. (The given concentrations are those in the reaction mixture, so syringe concentrations are double these values.) Both syringes contained 10 mM $MgCl_2$. The enzyme concentrations and time-sweeps are given in the figure.

the lag periods were greatly decreased (v_a values were directly proportional to enzyme concentration under the conditions of all the experiments described here, whereas there was an approximately inverse relationship between the length of the lag period and enzyme concentration).

3.2. *The role of FDP in shortening the lag periods*

In view of reports that FDP is an activator of the reaction [7] it seemed possible that the 'switching-on' of PFK activity observed in the time-courses of figs.1 and 2 could have resulted from a slow accumulation of small, activating amounts of FDP during the lag-period. In this case the lag period should be decreased by FDP. This decrease was observed in an experiment conducted under the conditions of fig.1, and the same effect, at a far greater enzyme concentration is shown by the stopped-flow time courses of fig.3. From the results of fig.3 it can be seen that there is no observable lag if FDP is included in the enzyme syringe or the substrate syringe, although the same value of v_a was observed with or without FDP.

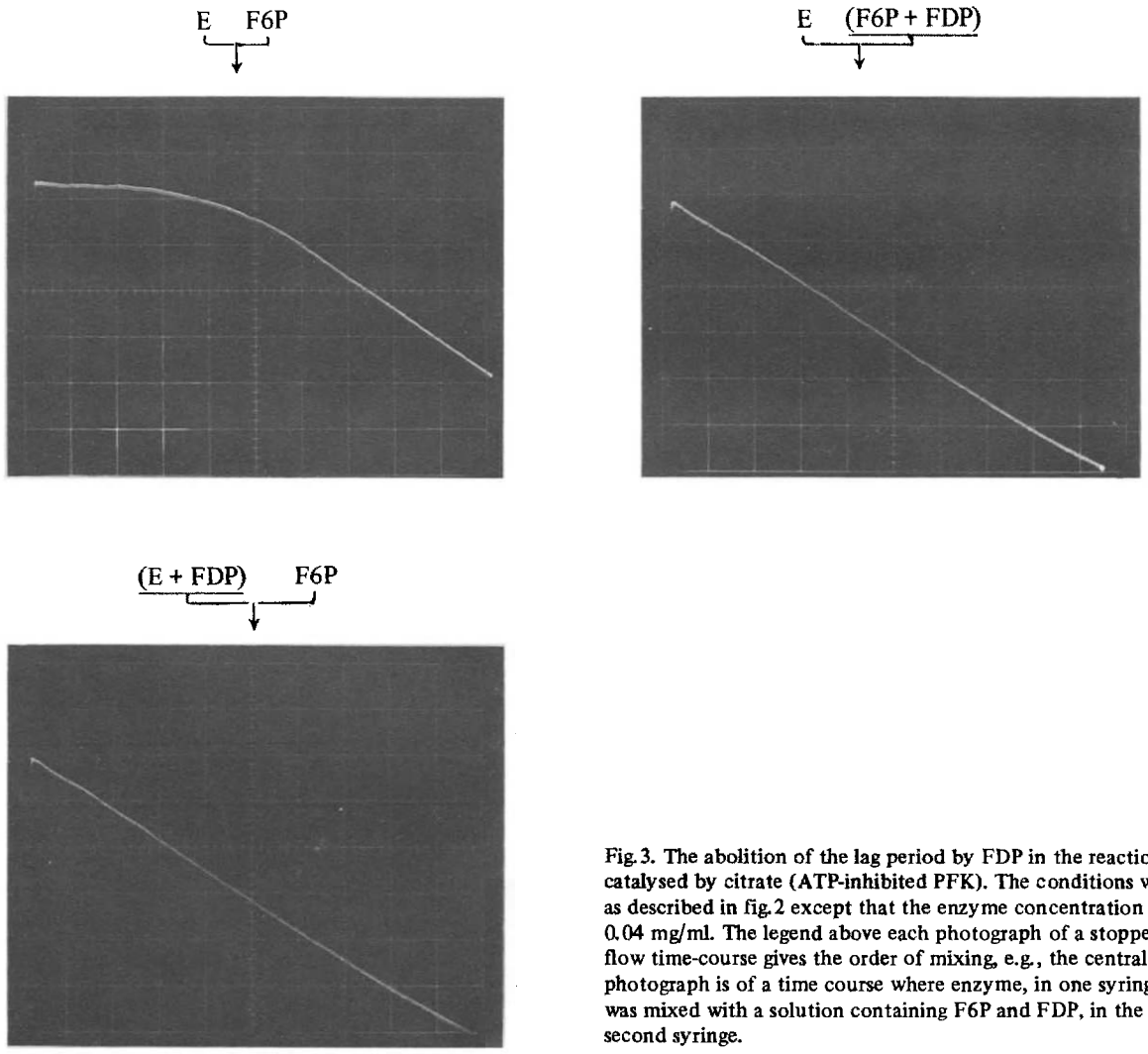

Fig. 3. The abolition of the lag period by FDP in the reaction catalysed by citrate (ATP-inhibited PFK). The conditions were as described in fig.2 except that the enzyme concentration was 0.04 mg/ml. The legend above each photograph of a stopped-flow time-course gives the order of mixing, e.g., the central photograph is of a time course where enzyme, in one syringe, was mixed with a solution containing F6P and FDP, in the second syringe.

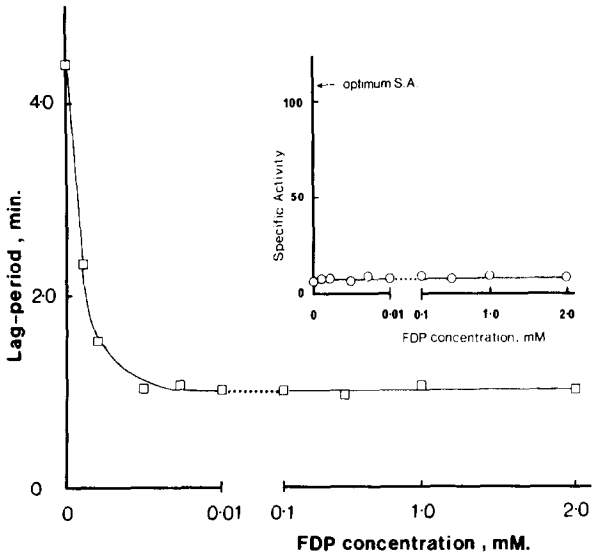

Fig.4. The effect of FDP concentration on the lag-period in time courses with ATP/citrate inhibited PFK. Time-courses for reactions with 7.2 μg/ml enzyme and 3 mM ATP, 1 mM citrate and 10 mM $MgCl_2$ were followed by using the spectrophotometric method described in Materials and methods and in fig.1. The reactions were initiated by addition of F6P to give a concentration of 0.4 mM. The inset shows the variation of the V_a value (given as specific activity, in units of μmol/min/mg PFK) with FDP concentration.

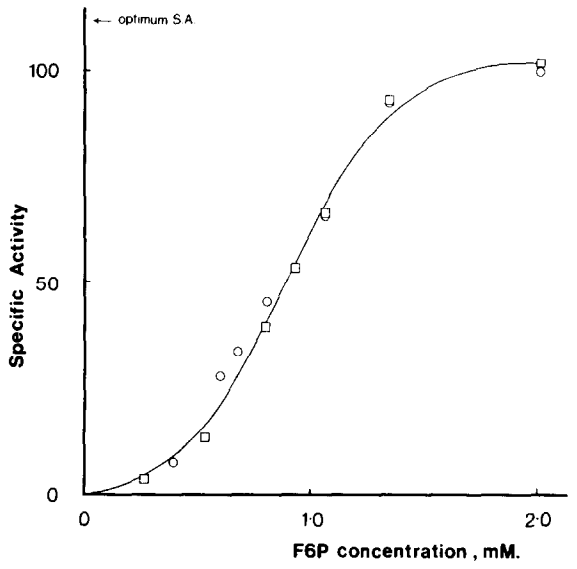

Fig.5. The velocity versus F6P concentration dependence of the reaction catalysed by PFK, in the presence and absence of FDP. Reactions were followed by using the spectrophotometric method described in Materials and methods and fig.1. Enzyme (5.2 μg/ml) was incubated in a solution containing 1.5 mM ATP, 0.4 mM citrate and 10 mM $MgCl_2$, and the reaction initiated by addition of F6P to the given concentrations. The instantaneous reaction rates in the presence of 0.1 mM FDP are given by the circles, and the v_a values, reached after a lag period in the absence of FDP, by the squares. The vertical axis is in units of μmol/min/mg.

From the time-courses of fig.3, as well as a number of other traces at different sweep speeds on the oscilloscope, it was possible to show that the half-life for the FDP-mediated activation of PFK was less than 0.4 sec (see Discussion).

The effect of FDP in reducing the lag period without changing the v_a value was investigated further by following time-courses of reactions in which enzyme was incubated with different concentrations of FDP together with the inhibitory ligands, before initiation of the reaction with F6P. It can be seen from the results of fig.4 that, although FDP reduced the lag period, even at a concentration of 2 mM it did not begin to overcome the 83% inhibition of the enzymic reaction by the concentrations of ATP and citrate in the assay mixture. A further demonstration of this effect of FDP in decreasing the length of the lag period without altering the v_a value is given in fig.5, which shows that the dependence of v_a on F6P concentration is the same in the presence and absence of 0.1 mM FDP.

The absolute value of the maximum specific activity recorded in fig.5 is important in that it indicates that there is not a large pool of inactive dimer present in these experiments.

The concentration of FDP required to reduce the lag period to a minimum was less than 5 μM (see fig.4); and the concentration to achieve half the effect was about 0.6 μM. Production of a concentration of FDP in this low range would not be detected under the conditions of the time-course shown in fig.1 since it represents only about 1% of the reaction. In addition, it should be noted that the length of the lag periods decreased with increasing concentrations of F6P (see fig.5).

Time-courses exhibiting lag-periods like that seen in fig.1 were also observed when the enzyme was preincubated with ATP alone (fig.6) and again FDP appeared to be the crucial factor in controlling the length of the lag, as evidenced by the increased lag periods

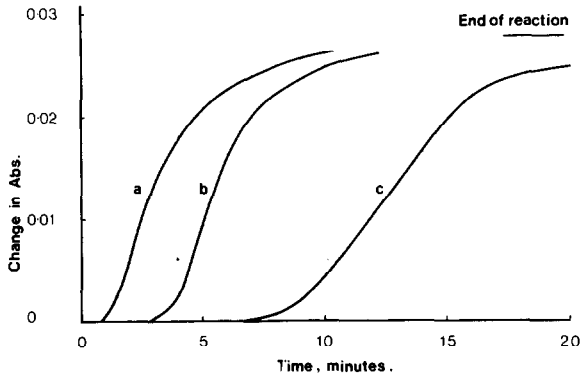

Fig.6. The effect of aldolase on time-courses of the PFK-catalysed reaction. PFK (5.1 μg/ml) was incubated in a solution containing 1.5 mM ATP and 1.4 mM $MgCl_2$ and the reaction initiated by addition of F6P to a concentration of 0.2 mM. The reaction was followed by using the standard spectrophotometric assay (see fig.1). The three time-courses are for (a) control, without aldolase (b) with 2.6 μg/ml aldolase (c) 3.9 μg/ml aldolase. The aldolase was added to the reaction mixture before addition of the F6P.

observed when aldolase was incorporated into the assay mixtures (see fig.6).

It can be seen from the time-courses of fig.6 that the v_a value is decreased at the highest concentration of aldolase. This illustrates the danger of using aldolase-coupled assays to follow the PFK-catalysed reaction since, although it is necessary to include sufficiently high concentrations of the coupling enzymes to ensure that the coupling step is not rate-limiting, such concentrations will decrease the observed rate.

4. Discussion

The foregoing results demonstrate that incubation of PFK with inhibitory concentrations of specific, allosteric ligands, before initiation of the enzymic reaction with F6P, gives time-courses with long lag-periods during which the enzyme appears to be inactive. At low enzyme concentrations these lag-periods can extend to many minutes, but even with concentrations of enzyme approaching that in vivo the lag-periods are still on the time-scale of seconds (e.g. see fig.2). We have also shown that, although FDP abolishes or greatly decreases the lag periods, it does not overcome completely the inhibition by a mixture of ATP and citrate under the assay conditions employed in this study. (i.e. the v_a value is not increased by FDP).

Other workers have also reported long lag-periods in the PFK reaction albeit at very low enzyme concentrations. Colombo et al. [6] observed lag-periods of up to 15 min in a reaction in which PFK was preincubated with 1 mM MgATP and 0.1 mM citrate before initiation of the reaction with F6P. However these authors also reported that the addition of FDP to the assay mixture, or increasing the concentration of aldolase and other coupling enzymes, did not increase the length of the lag, observations which were taken to demonstrate that the lag-periods did not result from the accumulation of low, activating concentrations of FDP during the initial stage of the reaction.

These findings are in disagreement with those of the present study and possibly reflect the differences in procedure: Colombo et al. [6] used an assay at pH 7.0 in which FDP production was coupled to NADH oxidation through the aldolase and glycerophosphate dehydrogenase-catalysed reactions, whereas we employed as indicator-coupled assay at pH 7.14. In addition the enzyme concentrations used by Colombo et al. [6] were about 0.3 μg/ml compared with 5 μg/ml or greater in the present work.

The use of an FDP-coupled assay to evaluate the effect of FDP on the enzymic reaction is clearly unsatisfactory, since the FDP would be removed from the system before it exerted the effect reported here. However, it is more difficult to account for the failure of Colombo et al. [6] to observe the aldolase-induced increase in the lag-period in view of the results of fig.6 as well as the observations by El-badry et al. [8] and Emerk and Frieden [9], that both aldolase and FDPase cause an inhibition of the PFK-catalysed reaction.

Colombo et al. [6] favoured the hypothesis that the lag-period originated from the preincubation of the enzyme with ATP and citrate giving an inactive, depolymerised form of the enzyme which, on addition of F6P, underwent a slow aggregation to give a new equilibrium between active and inactive conformers. This seems unlikely to be the cause of the present results because a simple polymerisation model, such as a combination of inactive dimers to give active tetramers, cannot give time-courses of the shape of those shown in figs.1 and 4 or indeed of the type presented by Colombo et al. [6]. This is because such a process would be second-order in dimer concentra-

tion so, unlike the time-courses in figs.1–3, the rate of appearance of product would increase rapidly at first but more slowly thereafter. We have confirmed that a simple polymerisation model cannot give curves of the shape of say, fig.1, by analytical, as well as computer-simulation studies (Hollaway, M. R. and Hood, K., unpublished work). In addition, the present results require that an aggregation of inactive dimers to give active tetramers would have a second order rate-constant of greater than $10^7 M^{-1}s^{-1}$, whereas available data indicate that this process is considerably slower (e.g. see refs. [10] and [11]).

Emerk and Frieden [9] also reported an acceleration in enzyme-initiated, ADP-coupled assays of PFK activity in the presence of P_i at pH 6.5, and have concluded that FDP is the critical factor in determining the rate of the reaction at this pH value. The present results indicate that FDP is also a crucial factor in determining PFK activity at the 'physiological' pH value of 7.1–7.2 (where the specific activity of the enzyme is at least ten times greater than at pH 6.5) and with concentration of PFK approaching that in vivo, i.e. about 0.3–0.5 mg/ml (see ref. [13]).

A simple model to account for the present results combines a slow generation of FDP in the quiescent period of the time-course (catalysed by a low residual enzyme activity), with an FDP-induced change in enzyme conformation (rather than aggregation state). The role of FDP is uncertain but two possible interpretations are: (a) that binding of FDP to some sites in a given PFK molecule causes a conformational change such that catalytic sites in other subunits within the molecule become functional, so giving rise to the strongly autocatalytic time-courses; (b) that the binding of FDP leads to an increase in the conformational mobility of the enzyme so that it more rapidly attains the distribution between different forms determined by the other ligands present, e.g. FDP could allow the activating influence of F6P to be expressed. In this model FDP can be considered to act as a hysteretic effector.

It is noteworthy that El-badry et al. [8] have reported that the activity of sheep heart PFK appears to be dependent on the presence of FDP and have pointed out the analogous role of NAD^+ in activating glyceraldehyde 3-phosphate dehydrogenase. The present results extend this parallel since the strongly autocatalytic progress-curves are similar in shape to those obtained by Trentham [14] in the reaction of NADH with 1,3-diphosphoglycerate catalysed by sturgeon muscle glyceraldehyde 3-phosphate dehydrogenase.

Whatever interpretation may apply, the fact remains that FDP is profoundly different from F6P in its capacity for activating PFK, e.g. 5 μM FDP is sufficient to saturate the activation process involving the abolition of the lag-period, under conditions where 400 μM F6P has little effect (see fig.4). It seems unlikely that a difference in ligand affinity could be the sole cause of this different capacity for activation since the values of the dissociation constants for F6P and FDP differ by less than 20-fold for PFK alone, and are closely similar in the presence of 5 mM citrate [12]. Therefore it is more likely that the binding of FDP of PFK induces a different conformational change from that caused by the binding of F6P, a conclusion which supports that of Kemp and Forest [4] who observed that FDP protects a different pair of slowly reacting thiol groups from the two protected by F6P binding.

The present study was carried out with the object of measuring the activity of PFK under conditions approximating those in vivo. In skeletal muscle the concentration of PFK is in the range of 0.1–0.5 mg/ml, the ATP concentration between 5 and 7 mM (see ref. [1]) and the citrate concentration about 0.5 mM (e.g. for rat gastrocnemius [15]). In resting, white, skeletal muscle the concentration of F6P and FDP would be expected to be low and hence PFK virtually inactive, as in the initial part of the progress-curves of fig.2. Thus the conditions for the time-courses of fig.2 resemble those in vivo; the initiation of the reaction by mixing with F6P would correspond to an increased level of F6P, arising in vivo indirectly from activation of phosphorylase by an increased Ca^{2+} concentration, or enhanced phosphorylase kinase activity. Thereafter, the acceleration of the PFK reaction would take place as FDP accumulated. Clearly, changes in vivo in adenine nucleotide levels would also influence PFK activity directly, as well as indirectly, e.g. by increased AMP concentrations inhibiting fructose-1,6-diphosphatase, thereby allowing FDP to accumulate.

It has been suggested that, in order to account for the extent of increase of glycolytic flux on initiation of muscle contraction, it is necessary to postulate a 'substrate cycle' at the level of the PFK and fructose-1,6-diphosphatase-catalysed reactions (see ref. [1]).

The present results show that an extensive and rapid change of PFK activity under conditions approximating those in vivo, can be obtained without the operation of such cycling. In this case it is possible that the role of fructose-1,6-diphosphate in white, skeletal muscle is to maintain a low FDP concentration whilst the muscle is resting, thereby giving a low PFK activity due to the strong inhibition by ATP and citrate.

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